Expanding the Genotypic Spectrum of *CCBE1* Mutations in Hennekam Syndrome

Joanna Crawford[1^], Neil Bower[1^], Benjamin Hogan[1], Ryan Taft[1,2], Michael Gabbett[3,4], Julie McGaughran[3,4*] and Cas Simons[1]

[1] Institute for Molecular Bioscience, The University of Queensland, Brisbane, QLD, Australia

[2] Illumina, Inc., San Diego, CA USA

[3]Genetic Health Queensland, Royal Brisbane & Women's Hospital, Brisbane, Australia

[4] School of Medicine, The University of Queensland, Brisbane, QLD, Australia

Running title

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^ These authors have contributed equally to this work

*To whom correspondence should be addressed:

A/Prof Julie McGaughran MD FRCP FRACP

Director Pre-eminent staff specialist

Genetic Health Queensland

Bldg C28, RBWH Campus

Herston QLD 4029

T +61 7 3646 1686 **F** +61 7 3646 1987

julie.mcgaughran@health.qld.gov.au

ABSTRACT

Hennekam lymphangiectasia–lymphedema syndrome is an autosomal recessive disorder, with 25% of patients having mutations in *CCBE1*. We identified a family with two brothers presenting with primary lymphedema, and performed exome sequencing to determine the cause of their disease. Analysis of four family members showed that both affected brothers had the same rare compound heterozygous mutations in *CCBE1*. The presumed paternally inherited NM_133459.3:c.310G>A; p.(Asp104Asn), lies adjacent to other known pathogenic *CCBE1* mutations, while the maternally inherited NM_133459.3:c.80T>C; p.(Leu27Pro) lies in the CCBE1 signal peptide, which has not previously been associated with disease. Functional analysis in a zebrafish model of lymphatic disease showed that both mutations lead to CCBE1 loss of function, confirming the pathogenicity of these variants and expanding the genotypic spectrum of lymphatic disorders.

Key words

Hennekam syndrome, lymphedema, CCBE1, exome sequencing

INTRODUCTION

Hennekam lymphangiectasia–lymphedema syndrome is characterized by generalized lymphatic dysplasia with malformations of the lymphatic system affecting various organs, including the intestinal tract, pericardium, and limbs. Facial dysmorphism and cognitive impairment are also features commonly associated with disease presentation [Hennekam et al., 1989]. Hennekam syndrome is a genetically heterogeneous disorder. Biallelic mutations in *CCBE1* (Hennekam lymphangiectasia-lymphedema syndrome 1 (HKLLS1; MIM:235510)) and *FAT4* (Hennekam lymphangiectasia-lymphedema syndrome 2 (HKLLS2; MIM:616006)) have been reported as causative, with approximately 25% of patients having mutations in *CCBE1* [Alders et al., 2014]. Collagen- and calcium-binding EGF domain-containing protein 1 (CCBE1) is indispensible for lymphangiogenesis during development in humans and model organisms. Hogan et al [2009a] identified a causative coding mutation in *ccbe1* using a forward genetic screen in zebrafish. The mutant, termed *full of fluid* (*fof*), lacked truncal lymphatic vessels including the thoracic duct, but retained normal blood vasculature.

The *CCBE1* mutations previously reported to be causative for HKLLS1 are predominantly missense mutations located in the protein functional domains (collagen domain or EGF domain) or cysteine rich regions upstream of the EGF domain [Alders et al., 2013]. Here we report two brothers suffering from a primary lymphedema with a clinical presentation consistent with Hennekam lymphangiectasia-lymphedema syndrome, in whom exome sequencing (ES) identified bi-allelic damaging variants in *CCBE1*.

CLINICAL REPORT

The family was referred when their second son presented with a protein-losing enteropathy. The parents were non-consanguineous and there was a healthy older brother. The

proband was born by a vaginal delivery at 41 weeks weighing 4,440 g. He presented at 2 weeks of age. He had hypoalbuminemia at 3 weeks of age and some dysmorphic features. The finding of asymmetrical variable edema of both hands and feet after normalization of his serum albumin supported a diagnosis of intestinal lymphangiectasia. His younger brother was also affected by the condition. He was delivered at 38 weeks gestation after a pregnancy complicated by polyhydramnios. His birth weight was 3,320 g and he was noted to have an undescended testis and pedal edema. He was admitted to hospital at day 10 with hypoalbuminemia associated with diarrhea and poor feeding. He was noted to have gastro-intestinal reflux. He was assumed to have the same diagnosis as his brother. He also had problems with upper airway obstruction. He had variable inspiratory and expiratory stridor from birth. Bronchoscopy demonstrated the presence of tracheobronchomalacia. The first affected boy had no intellectual problems, however the second boy had developmental delay, a sensory processing disorder and had some seizures. Both brothers presented with similar facial features. Each had round faces with widely spaced eyes. They had depressed nasal bridges with bilateral epicanthus, long philtrums and thin upper vermilion. The features were consistent with a diagnosis of Hennekam syndrome.

METHODS

This study was approved by the Royal Brisbane and Women's Hospital Human Research Ethics Committee, the Royal Children's Hospital Human Research Ethics Committee and the University of Queensland Medical Research Ethics Committee. Informed consent was obtained from the three siblings and their mother prior to initiation of this study. Peripheral blood was collected from all individuals and genomic DNA extracted using standard procedures.

DNA library preparation, exome enrichment, sequencing, read alignment and variant calling were all performed as described [Simons et al., 2015]. Subsequent analysis and

identification of candidate variants was performed with an in-house workflow incorporating the annotated variant data and pedigree information. Targeted exons were sequenced to an average depth of at least 100X in all four family members.

Intronic primers generating amplicons spanning exons 1 and 4 of *CCBE1* were bi-directionally Sanger sequenced to respectively identify, and confirm identified, variants with reads aligned to the reference human genome (GRCh37).

The coding sequence of human *CCBE1* was cloned into the pCS2+ vector and site directed mutagenesis performed to introduce p.Leu27Pro and p.Asp104Asn variants. Capped *CCBE1* wildtype and variant mRNA were transcribed and 350 pg was injected into the yolk of single cell stage embryos as described [Hogan et al., 2009b]. The *ccbe1* morpholino was injected as described [Hogan et al., 2009a]. Thoracic duct formation was quantified as present or absent in each body segment (somite) across six segments at 5 days post fertilization (dpf). For simplicity a score of thoracic duct present (TD+) or thoracic duct deficient (TD-) was determined based on the presence or complete absence of TD fragments, respectively. Live zebrafish embryos were mounted in 0.5% low melting agarose laterally and imaged using a Zeiss LSM 710 FCS confocal microscope. Images were processed using ImageJ 1.47 software (National Institute of Health).

Primer sequences for Sanger sequencing, *CCBE1* cloning and mutagenesis are available upon request.

RESULTS

Genomic DNA was provided for four family members, including the mother, two affected male children, and one unaffected male sibling. No paternal DNA sample was collected.

Both affected individuals shared the same two rare missense variants in *CCBE1*. Initial analysis of ES data identified a single potentially pathogenic variant in *CCBE1*, NM_133459.3: c.310G>A; p.(Asp104Asn), that occurs in a conserved residue in the cysteine rich region amino-terminal to the EGF functional domain. This variant is present in dbSNP 142 (rs139165727) and is reported in ExAc as restricted to the European (Non- Finnish) population with an allele frequency of 0.0003 (with no homozygous individuals reported). It is predicted to be damaging by Polyphen and neutral by SIFT. The CCBE1 Asp104 residue is conserved, and is invariant in all sequenced vertebrates. Both affected siblings were heterozygous for the c.310G>A variant, as was the unaffected sibling. The mother was wild type at this position. The second variant was initially undetected by exome sequencing due to poor coverage of the 5' end of the gene. Sanger sequencing was then conducted of exon 1 and identified the NM_133459.3: c.80T>C; p.(Leu27Pro) change. This variant was absent from both dbSNP 142 and ExAc. The CCBE1 Leu27 residue is a conserved residue in the signal peptide sequence, a domain important for the extracellular secretion of the protein. This residue is invariant in all mammals and marsupials, with the p.(Leu27Pro) change predicted to be probably damaging by SIFT and Polyphen. The c.80T>C variant was inherited from the mother, was present in both affected brothers, and was not present in the unaffected sibling. The genotypes and the segregation of both *CCBE1* variants were confirmed in all individuals by Sanger sequencing. The *CCBE1* variants identified are consistent with bi allelic loss of function in CCBE1.

To confirm that the two variants are deleterious to CCBE1 activity, we used a zebrafish model to perform a rescue assay for CCBE1 loss of function. The Asp104 residue is conserved between human and zebrafish, however there is considerable sequence divergence in the signal peptide at the site of the Leu27Pro variant (Figure 1). For this reason, we used overexpression

of human *CCBE1* mRNA for the rescue assay. Zebrafish embryos were injected with either a previously validated morpholino (MO, modified anti-sense oligomer) targeting the zebrafish *ccbe1* homologue alone [Hogan et al., 2009a; Alders et al., 2009], or the MO in conjunction with human *CCBE1* mRNA.

Injection of the *ccbe1*-targeting MO alone, resulted in the loss of lymphatic development. Co-injection of wild-type human *CCBE1* mRNA with MO rescued the lymphatic defect in the majority of embryos tested. However, co-injection of either the p.Leu27Pro or p.Asp104Asn mutant *CCBE1* mRNA failed to rescue lymphatic development in any embryo tested (Figure 2). These results demonstrate that both the Leu27Pro and Asp104Asn variants lead to loss of function of CCBE1.

DISCUSSION

Primary lymphedema is a chronic, genetically determined, heritable disorder of incorrectly developed vasculature that results in fluid (lymph) accumulation, particularly in the extremities. Mutations in nine genes have been identified in different forms of primary lymphedema, all of which appear to be involved in the vascular endothelial growth factor-c/vascular endothelial growth factor receptor-3 signaling pathway [Alders et al., 2013; Mendola et al., 2013], a pathway shown to be essential for correct lymphatic vessel formation during embryonic development [Alitalo 2011].

Of the various forms of primary lymphedema, *CCBE1* mutations appear to be predominantly causative for Hennekam Syndrome [Alders et al., 2013]. Fewer than 50 patients with Hennekam Syndrome have been reported in the literature to date, with 25-29 % of those having biallelic *CCBE1* coding mutations [Alders et al., 2014; Jackson et al., 2016] and 20% having biallelic *FAT4* mutations. Fotiou et al [2015] recently reported biallelic mutations in

PIEZO1 can result in generalized lymphatic dysplasia, phenotypically similar to Hennekam Syndrome.

The disease presentation in patients with Hennekam Syndrome is quite variable and, interestingly, does not appear to be indicative of the genotypic basis of the disorder [Alders et al., 2013]. Patients in whom pathogenic *CCBE1* mutations are found are no exception, with Alders et al [2013] describing considerable phenotypic variation between individuals who had the same *CCBE1* mutations. Here we present clinical reports for two affected brothers, both of whom had the same biallelic *CCBE1* missense mutations, but also show phenotypic variation. In this family, the presentation of lymphedema and the facial dysmorphism characteristic of the disease was consistent between the boys, but the intellectual phenotype showed considerable intra-familial variation.

Interestingly, neither of the mutations identified in the patients reported here were in functional domains, nor are they predicted to alter cysteine residues, and as such do not typify the *CCBE1* mutations published to date. The p.(Asp104Asn) mutation we report here has previously been identified in conjunction with the common NM_133459.3:c.223T>A; p.(Cys75Ser) mutation in compound heterozygous siblings who presented with a mild phenotype [Connell et al., 2012], however this is the first report of a causative mutation in the signal peptide domain responsible for correct CCBE1 localization.

To establish the deleterious nature of these mutations we employed a zebrafish lymphangiogenesis model that has been used for testing the pathogenicity of *CCBE1* mutations identified in patients with HKLLS1 [Alders et al., 2009]. In this work, the authors assessed the pathogenicity of their *CCBE1* mutations using the zebrafish equivalent of the human mutations. In the present study, as the Leu27 amino acid in the signal peptide is not conserved between

zebrafish and human, this prohibited the use of zebrafish mRNA for rescue experiments. We therefore introduced the variants into human *CCBE1* and used human *CCBE1* mRNA to perform the rescue experiments. The robust rescue we observed upon human *CCBE1* mRNA injection into zebrafish *ccbe1* MO embryos demonstrated that the human protein was functional in zebrafish and opens up new possibilities for using the zebrafish system for structural and functional assays as well as future therapeutic assays reliant upon using the human form of CCBE1.

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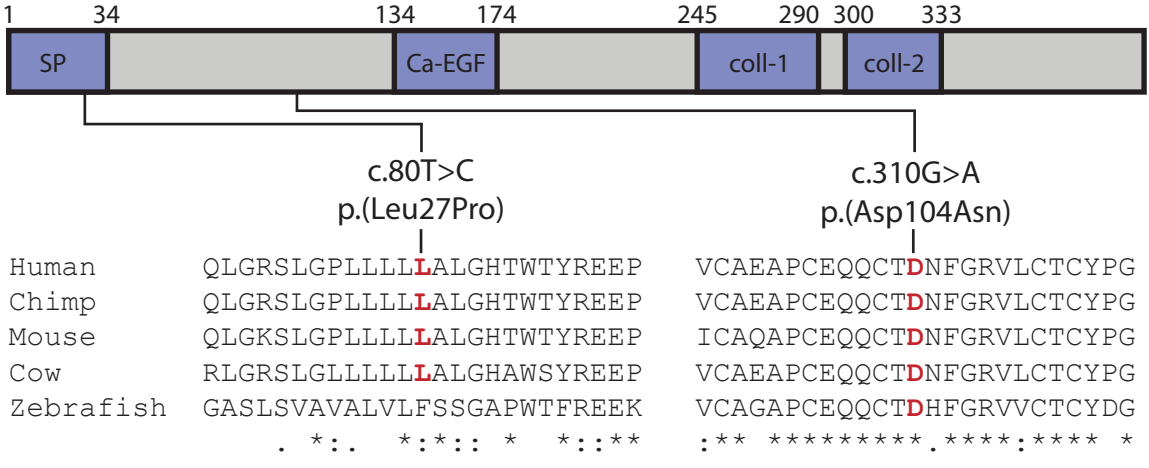
FIGURE LEGENDS

Figure 1: Schematic representation of the functional domains of CCBE1 (obtained from UniProt). The signal peptide (SP), calcium-binding EGF (Ca-EGF) domain, collagen repeat 1 (coll-1), and collagen repeat 2 (coll-2) are indicated. Patient mutations and evolutionary conservation at those residues are depicted.

Figure 2: Functional analyses of the human CCBE1 mutations in the *ccbe1* zebrafish model using transgenic line *TG(fli1a:gfp)*^{y1}, *TG(kdr-l:ras-cherry)s916*. *Fli1a* (green) is expressed in both blood and lymphatic vessels, whereas *kdr-l* (red) is only expressed in blood vessels [Hogan et al., 2009a]. At 5 d post fertilization the thoracic duct (arrow) is present in a) control embryos, but is absent (*) in b) embryos injected with *ccbe1* ATG-targeting morpholino. The *ccbe1* ATG MO phenotype is rescued by injection of c) wild-type human *CCBE1* mRNA, but not with human mRNA encoding the d) p.Leu27Pro (L27P) or e) p.Asp104Asn (D104N) substitutions. f) Summary of rescue experiments evaluating the presence/absence of the thoracic duct (TD).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SP
Ca-EGF
coll-1
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c.80T>C
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c.310G>A
p.(Asp104Asn)
Human QLGRSLGPLLLLLALGHTWTYREEP VCAEAPCEQQCTDNFGRVLCTCYPG
Chimp QLGRSLGPLLLLLALGHTWTYREEP VCAEAPCEQQCTDNFGRVLCTCYPG
Mouse QLGKSLGPLLLLLALGHTWTYREEP ICAQAPCEQQCTDNFGRVLCTCYPG
Cow RLGRSLGLLLLLLALGHAWSYREEP VCAEAPCEQQCTDNFGRVLCTCYPG
Zebrafish GASLSVAVALVLFSSGAPWTFREEK VCAGAPCEQQCTDHFGRVVCTCYDG
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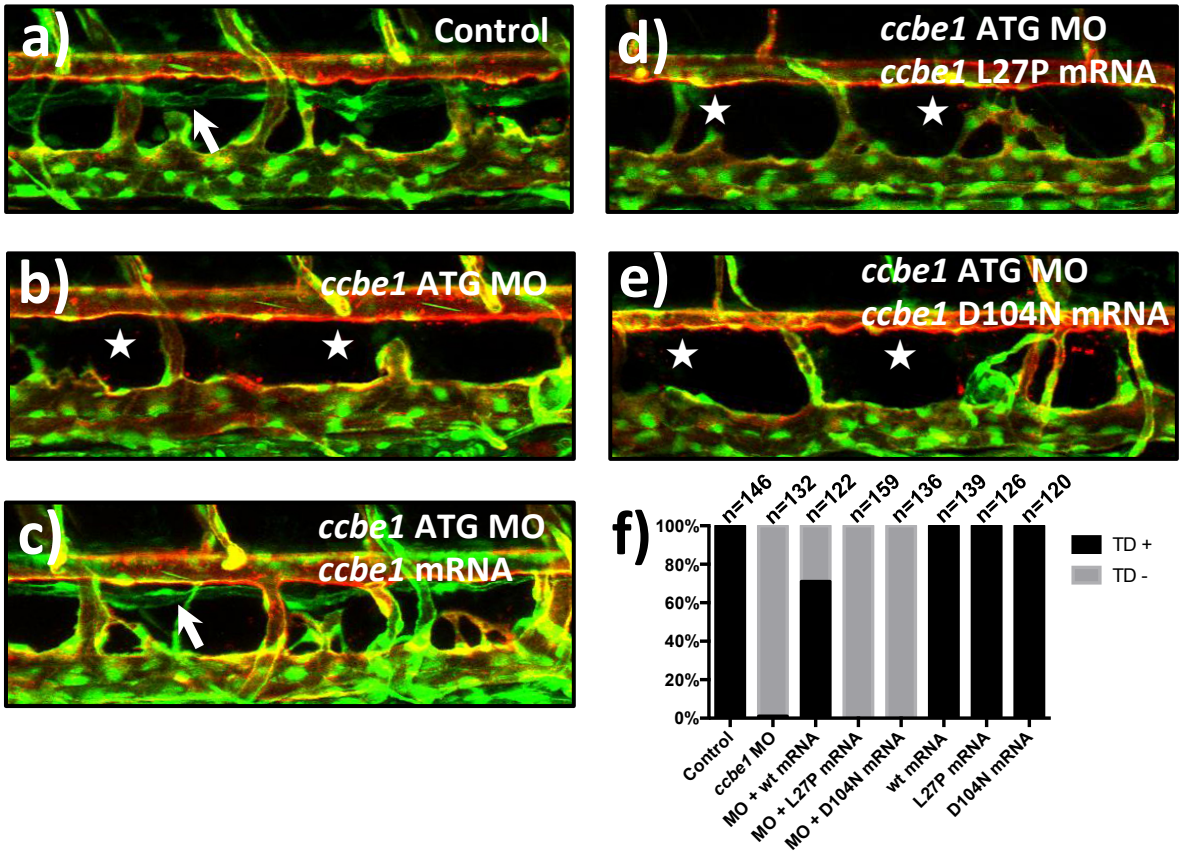
a)
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ccbe1 ATG MO
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ccbe1 D104N mRNA
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n=122
n=159
n=136
n=139
n=126
n=120
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Control
ccbe1 MO
MO + wt mRNA
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